The Elliptic Phase Diagram of Proteins: What is Left Out?

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Received 21 December 2006/Accepted 28 December 2006

Abstract
This review paper emphasizes the role of the changes in the thermal expansion, the heat capacity and the compressibility of protein solutions in the temperature and pressure dependence of the unfolding of proteins and some water soluble polymers. These parameters need further detailed interpretation from computer simulations in order to advance our understanding of the stability diagrams of proteins.

Keywords: proteins, thermal expansion, compressibility, heat capacity.

1. Introduction

The stability of proteins in aqueous solutions as a function of temperature and pressure has been found to show the shape of an ellipse. This is quite different for molecules of small molar mass such as nutrients, colours and flavours, and this is the basis for the application of high pressure in food science [1].

The physical basis for the elliptic equation comes from the temperature and pressure dependence of the volume changes of the unfolding of the protein under a given solvent and pH condition. Maxwell thermodynamic relations give also the temperature dependence of the entropy change upon unfolding. This brings us to the well known observation that the enthalpy change upon unfolding is temperature dependent [2,3].

In this paper we draw attention to the quantities that have been less considered in the general literature: the thermal expansion, the compressibility and the heat capacity of proteins and the relation between these quantities e.g. via the Grüneisen parameter [4]. These parameters contain useful information on the thermodynamic fluctuations of the system [5]. These quantities are usually “left out” of the molecular interpretations.

The etymological origin of the word ellipse (from the Greek elleipsis: what is left out) is usually associated with the theory of the conic sections. But this does not explain the origin of the word. For reasons that are not entirely clarified the name originates from the comparison of surfaces, which is clear from the quadratic nature of the equation [6].

2. Thermodynamics and kinetics

2.1 Thermodynamics

Mathematically the elliptic shape originates from the fact that in a Taylor expansion of the free energy difference between the denatured (\(G_D\)) and the native state (\(G_N\)), \(\Delta G = G_D - G_N\), as a function of temperature and pressure, the second order terms give a significant contribution as shown in the following expression [2]:

\[
\Delta G = \Delta G_D - \Delta G_N = \sum_{i=1}^{\infty} \left[ \left( \frac{\partial^2 \Delta G}{\partial T^2} \right)_P \frac{T^2}{2} - \left( \frac{\partial^2 \Delta G}{\partial P^2} \right)_T P^2 \right]
\]
Fig. 1 Schematic representation of the stability diagram of a protein. Similar diagrams are observed for the coil-globule transition of water soluble polymers and the inactivation kinetics of enzymes and microorganisms. Note the thermodynamic similarities between the cold and pressure denaturation that are in contrast with the temperature denaturation. It can be seen that, at low pressure, the protein is more resistant to temperature unfolding. There are a few proteins that show the opposite behavior, i.e. they are destabilised by pressure.

\[
\Delta G(p,T) = \Delta G_0 - \Delta S_0(T - T_0) + \Delta V_0 (p - p_0) \\
+ \Delta C_p ((T - T_0) - T \ln(T/T_0)) - (\Delta \beta/2) (p - p_0)^2 + \Delta \alpha (T-T_0) (p - p_0)
\]

where \( \Delta G_0, \Delta V_0 \) and \( \Delta S_0 \) refer to the reference conditions \( p_0 \) and \( T_0 \). The second order terms, \( \Delta \alpha \) \( (=\partial \Delta V/\partial T)_p \), \( \Delta \beta \) \( (=\partial \Delta V/\partial p)_T \) and \( \Delta C_p \) \( (=T (\partial \Delta S/\partial T)_p) \), are proportional to the differences in thermal expansion factor, compressibility factor and heat capacity between the denatured and the native state of the protein, respectively.

These quantities are of particular interest as they can be related to the changes in fluctuations in volume, energy and the cross-correlation of volume and energy, respectively. Such fluctuations underlie the dynamic behaviour of proteins in water [5].

An important issue is the shape of the elliptical diagram as shown in Figure 1. In the region where \( \Delta H \) and \( \Delta V \) are both negative, an increase in temperature will stabilize the protein against pressure denaturation. In the region where \( \Delta H \) is positive and \( \Delta V \) is negative, an increase in temperature lowers the denaturation pressure, and vice versa. In the region where \( \Delta H \) and \( \Delta V \) are positive, it can be seen that increasing pressure stabilizes the protein against thermal denaturation. Note, however, that this pressure-induced thermal stabilization is not observed for all proteins [7].

The signs of \( \Delta V \) and \( \Delta H \) provide a thermodynamic basis for the mechanistic and conformational differences between the pressure and heat denaturation of proteins, and rationalizes the similarities between the pressure and cold denaturation [8]. It is important to note that similar differences are observed for the coil-globule transitions of water soluble polymers [9].

Finally, it is of interest to consider the effect of the solvent and the charge of the protein on the position and shape of the phase diagram. The presence of co-solutes such as sucrose stabilize the protein against thermal as well as pressure unfolding [10,11]. Urea on the other hand destabilizes the protein with respect to temperature and pressure unfolding [11]. Similar effects have been observed for the behaviour of water soluble synthetic polymers [12]. Other factors such as macromolecular crowding have been virtually left unexplored with respect to pressure stability.

2.2 Kinetic aspects of the phase diagram

In many instances, the rate of denaturation as a function of pressure and temperature is studied yielding a \( p,T,k \)-diagram, where \( k \) is the rate constant of inactivation or denaturation. A mathematical analysis of the isokineticity curves yields the activation parameters for the
denaturation. These data are of considerable importance for applications in the food industry [13]. Similar to the \( p,T \)-phase diagram the \( p,T,k \)-diagram can be divided in three regions based on the signs of \( \Delta V^\# \) and \( \Delta H^\# \). The fact that the plots for the kinetic data show strong similarities with the thermodynamic data strongly suggests that the kinetic data are coupled to the thermodynamics of protein unfolding.

### 2.3 The mechanism of pressure denaturation

Suzuki [14] found that at temperatures below 30\(^\circ\)C the kinetics of the pressure denaturation of carbonylhemoglobin and ovalbumin was characterized by a negative activation enthalpy. Such negative activation energies have also been observed for the urea-induced denaturation of proteins. To explain his observations he proposed the following mechanism:

\[
P + nH_2O \rightleftharpoons P(H_2O)_n \rightleftharpoons P_D
\]

where \( P \) is the native protein, \( P(H_2O)_n \) is the hydrated protein and \( P_D \) the denatured protein. Thus, he suggested that pressure induces the penetration of water into the protein in a strongly exothermic step. That the mechanism for temperature denaturation is quite different can be concluded from the endothermic nature of the process.

Computer simulations on bovine pancreatic trypsin inhibitor [15] have provided further evidence for this water penetration mechanism showing that, under pressure, protein-protein hydrogen bonds are replaced by protein-water hydrogen bonds.

Pressure-induced protein denaturation is associated with volume changes in the order of -10 to -100 ml/mol. What is the origin of the volume decrease? The volume of a protein in solution, \( V_i \), is the sum of:

\[
V_i = V_{atom} + V_{cavities} + \Delta V_{hydration}
\]

where \( V_{atom} \) and \( V_{cavities} \) are the volumes of the atoms and the cavities (that originate from imperfect packing of the atoms in the native conformation), respectively, and \( \Delta V_{hydration} \) is the volume change resulting from the interactions of the protein with the solvent. Upon protein denaturation the volume of the atoms will not change, so the volume change accompanying the denaturation can be written as:

\[
\Delta V = \Delta V_{cavities} + \Delta \Delta V_{hydration}
\]

Contributions to \( \Delta \Delta V_{hydration} \) is usually attributed to the exposure of charged and hydrophobic groups to water which will cause a volume decrease.

The elimination of cavities upon denaturation is also expected to contribute to the observed negative volume change. This has been confirmed experimentally. Mutants of RNase A, in which the mutations created additional cavities, are characterized by a larger negative \( \Delta V \) upon denaturation [16].

Summation of the above contributions would result in a large and negative \( \Delta V \). However, experimentally only small, negative volume changes are observed. This suggests that there is also a positive contribution that, at least in part, compensates for the above negative contributions. The origin of this contribution is still the subject of debate [17].

The above described pressure-denaturation mechanism should also provide a basis for the observed differences between the heat and pressure denatured states [8].
3. Role of water and cavities

From the above described mechanism it is clear that water should be considered as a reaction partner rather than as an inert background. All protein processes are water embedded. This is supported by a number of observations. For instance, the presence of water is required for enzyme activity and it strongly affects the temperature stability of proteins. This has been observed for several proteins and it applies also to the pressure denaturation [19]. The resistance of bacterial spores and small organisms, such as tardigrades, and the stability of amyloid fibrils are other illustrations of the importance of water in the effects of pressure on organisms and molecules.

There is an increasing interest in the study of the thermal expansion [11], compressibility [20,21] and heat capacity [22] in an attempt to obtain more quantitative information on the role of hydration in various biological processes. The number of assumptions that are usually made are quite important as may be shown for compressibility data obtained from ultrasound velocity data [23]. Decomposition of the experimental data into intrinsic and hydration shell contributions is also based on a number of assumptions [24]. All this puts strong restrictions on the possibility to obtain information on the coupling between bulk water, hydration water and protein conformation.

Another approach is the study of the cavities and their volume changes with temperature and pressure. This may be done by following the intrinsic phosphorescence lifetime of chromophores inside the protein [25]. Another approach is positron annihilation lifetime spectroscopy. Experimental data on lysozyme show a temperature independence of the lifetime, an effect that has also been observed in water soluble polymers. Whereas pressure decreases the lifetime in lysozyme, no data are available for water soluble polymers [26].

If we can correlate the thermal expansion of water as given in Figure 2 with the stability diagrams from Figure 1, then it seems that the dominating factor for the explanation of the shape of the diagrams is the thermal expansion of the system. This then points to the crucial role of the tetrahedral network that is created in these systems from the strong and directional hydrogen bonding. This open network would also be largely responsible for the role of the cavities and their contributions to the volumetric properties of these systems. Computer simulations are the only means to get a deeper understanding of the contributions to these properties [11,27].

![Fig. 2 The thermal expansion of water as a function of temperature and pressure based on the NIST Standard Reference Database [18]. If the changes in the thermal expansion of water at given temperatures are plotted as a function of pressure, a diagram similar to the stability diagrams of proteins and polymers is obtained as shown schematically in Figure 1.](image)
4. Phase diagram: unfolding and aggregation

Pressure induced unfolding of monomeric proteins is usually observed in the range between 400-800MPa. The interesting differences that have been observed between the effect of temperature and pressure on the unfolding of proteins [8] and water soluble polymers [9] needs further detailed investigation. Also, the observed low pressure induced stabilisation of the temperature induced unfolding as in the case of chymotrypsinogen and the destabilisation observed for ribonuclease remain unexplained in terms of the properties of protein structure. The different characteristics of the heat and pressure denatured states will also affect properties such as the aggregation propensity and the gelation of proteins. The latter is being exploited in the food industry to produce foodstuffs with novel properties.

More recently, the ability of high pressure to dissociate protein assemblies has been applied to larger amorphous and fibrillar aggregates. It has been found that aggregates, such as inclusion bodies, can be dissociated by pressure under solution conditions where the monomeric protein is in its native conformation. Hence, it is possible to rescue these proteins from an aggregate and refold them, an observation that has interesting industrial and biotechnological applications. Amyloid fibrils, involved in a number of debilitating diseases, such as Alzheimer’s disease and transmissible spongiform encephalopathies, have also been shown to be sensitive to pressure, at least in the early stages of their formation. However, as aggregates are left to mature, they become more and more pressure insensitive [28]. One possible model that explains this stability is the β-helix which occurs in certain enzymes that are extremely pressure resistant [29]. The mature aggregates are also extremely temperature stable ($T_m>100^\circ C$), which has been attributed to the anhydrous nature of the fibrillar aggregates. In this respect they resemble bacterial spores. The pressure stability of amyloid fibrils has been discussed at length by Meersman and Dobson [30]. In addition, an increasing number of examples suggest that aggregation may also occur at high pressure, but the aggregate presumably has different characteristics from the conventionally heat-induced aggregates [31].

5. Conclusion

The conclusion from the present paper can be summarized as a further need for our understanding of water embedded interactions in biological systems. The thermal expansion, compressibility and heat capacity need further detailed interpretation from computer simulations. The relation between the shape of the stability diagram and possible events of the unfolded protein (gelation versus fibril formation) will certainly remain a topic of fascinating investigations for the future.

6. Acknowledgements

It is a pleasure to acknowledge the contributions of Dr. L. Smeller (Budapest) and Dr. F. Meersman (Leuven) to the work discussed in this paper. Our research is supported by FWO Research Foundation - Flanders.

7. References


